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HOW DOES APOPTOSIS OF OOCYTES AND GRANULOSA CELLS DUE TO CIGARETTE SMOKE EXPOSURE TO MICE BALB/C ? : EXPRESSION SMAD3, GDF9, APOPTOSIS

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ABSTRACT

Cigarette smoke contains harmful toxins for body, including nicotine, tar, and carbon dioxide gas which are carcinogens. This content is harmful for health of active and passive smokers, including disturbances in the reproductive system, hormone secretion disorders, and impotence. There are still no studies yet that explain the effect of cigarette smoke on female reproduction in the ovaries, namely granulosa cells and oocytes. to explain the effect of cigarette smoke on damage to oocytes and granulosa cells in follicles of ovaries in balb/c mice exposed to cigarette smoke. Results showed that the statistical test Mann Whitney of cigarette smoke increased the apoptotic index of granulosa cells (p-value 0.000 <0.05), and the results of the Independent T-Test statistical test showed that exposure to cigarette smoke decreased Smad 3 expression in granulosa cells (p 0.000 <0.05) in cigarette smoke and significantly lowered Smad 3 expression (p: 0.000) and also cigarette smoke significantly decreased GDF 9 expression (p: 0.000). Cigarette smoke did not affect the levels of the oocyte apoptosis index (p: 1).

Keywords: oocyte, granulose, smad 3, GDF9, apoptosis.

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INTRODUCTION

Cigarette smoke contains harmful toxins for body, including nicotine, tar, carbon dioxide gas which are carcinogens[1]. This content is harmful for health of active and passive smokers, including disturbances in the reproductive system, hormone secretion disorders, and impotence. The nicotine contained in cigarette smoke is one of the ingredients that increase free radical levels in the body. Free radicals cause an increase in ROS compounds in the body[2,3,4]. Smoking affects various metabolic and biological processes in the body including hormone secretion, this is due to the increased effect of oxidative stress due to the increase of free radicals in the body caused by cigarette smoke and blood serum. It will also affect the production of FSH and LH by the pituitary in the brain[3]. Cigarettes are a process of tobacco using additional ingredients or not. Cigarettes with added ingredients are called kretek cigarettes while cigarettes without any addition are called white cigarettes. In smoking process, there are two reactions, namely the combustion reaction and the pyrolysis reaction. The combustion reaction with oxygen will form CO2, H2O2, NO, SO, and CO compounds[5]. Pyrolysis reactions are reactions that break down chemical structures into many chemical compounds with complex structures[---]. Cigarette smoke enters the respiratory tract. NOX is an enzyme in all cell membranes. Hot steam contained in cigarette smoke that enters the air then will react with NOX become NADPH oxidase and $O2^{-}$ (superoxide). By SOD, the superoxide radical (O2-) is catalyzed into hydrogen peroxide (H2O2) and oxygen (O2). H2O2 is systemic oxidant that can affect all cells in the body[5,6.] H2O2 which is increased due to cigarette smoke will also penetrate the ovaries and affect granulosa cells. In granulosa cells, estrogen is produced. Estrogen in granulosa cells will affect the growth and development of oocytes through transcription activity by SMAD3. After activation of estrogen **receptors**[7,8,9,10] H2O2 in cells can damage DNA, fat, and protein. Smad 3 is protein that mediates the transcriptional activity of estradiol in granulosa cells that will affect the development of oocytes after activation of estrogen receptors[11.] If Smad 3 protein synthesis is inhibited, it cannot activate estrogen so that it cannot affect the growth and development of granulosa cells, even though granulosa cells are one of the cells that play a role in folliculogenesis. If granulosa cells do not develop, then folliculogenesis will also be disrupted[12]. H2O2 will also affect the proteins found in oocyte cells. In oocytes, there is GDF-9 which is glycoprotein secreted by oocytes, and an important factor for oocyte growth. besides that GDF9 can stimulate granulosa cell proliferation. The deficiency of GDF-9 will cause the development of follicles stopped, there is no cells around the follicle, and ability of the oocyte to divide miosis will be reduced.[13.14].

Smoking can also cause oxidation of glutathione (GSH, an antioxidant that protects DNA from damage caused by ROS), lowers blood levels of antioxidants, and increases the release of superoxide radical[15]. If the low GSH and GSSG increased, the oxidant H2O2 increased, then it can damage the cells that will undergo apoptosis protein[16.]GSH functions as anti-apoptosis, if GSH is low, then BAX will increase, Bcl2 decreases, Cyt c is oxidized and released, releasing caspase 9 and caspase 3 there will be apoptosis. But if the GSH is high, then the BCL2 increases, the cyt c will not be oxidized and released so that there is no apoptosis.[17]

MATERIALS AND METHODS

The type of research was analytically using *True Experimental* design with *post-test control group design.* Using sample of 20 female Balb/c mice (Mus Musculus) Balb/c aged around 8-10 weeks with bodyweight of 25-30 grams. The independent variable was exposure to cigarette smoke, while the dependent variable was Mad 3, GDF9, granulosa cell apoptosis, and oocyte apoptosis. This study was divided into 2 groups: Group 1: control group without exposure to cigarette smoke, Group 2: treatment group with exposure to cigarette smoke a dose of 1 cigarette per day for 20 days using a smoking pump. This research was conducted in Embryology Department, Faculty of Veterinary Medicine, Airlangga University. Data analysis used *independent t-test* or *Mannwithney*.

RESULTS AND DISCUSSION

Examination of Smad 3 expression in granulosa cells, GDF 9 in oocytes, using the IHC method. Examination of granular cell apoptosis and oocyte apoptosis using the TUNEK Essay method. The results showed that exposure to cigarette smoke can decrease Smad 3 expression in granulosa cells, decreased GDF9 expression in oocytes, increased granulosa cell apoptosis, but did not affect oocyte apoptosis. The results of the examination are listed in the table 1.

Table 1. Smad 3 expression, GDF9 expression, granulosa cell apoptosis, and oocyte apoptosis in the ovary

Variables	Group	Mean	SD	Min	Max	p
Smad 3	control	11.02	1.8582	9.2	15.7	
	treatment	0.59	0.2183	0.2	0.8	0.000
Granulosa cell apoptosis	control	0.00	0.00	0.00	0.00	
	treatment	0.82	0.14	0.60	1.10	0.000
GDF9	control	33.19	5.5	25.14	46.00	
	treatment	4.40	1.56	2.16	6.50	0.000
oocyte apoptosis	Control	0	0	0	0	
	treatment	0	0	0	0	1

Comparison of the mean results of Smad 3 and GDF9 expression between the control and treatment groups. Table 1 showed the mean Smad 3 and GDF9 expressions for each group. The lowest mean of Smad 3 and GDF9 expressions were found in the treatment group, while the highest mean was found in the control group. In the data normality test using *Shapiro Wilk*, it was found in the control group that data was normally distributed (p = 0.110) and treatment group data was normally distributed (p = 0.321). Then proceed with homogeneity test of the data using *Levene test*, and data was homogeneous (p = 0.06). The results of statistical tests using independent t-test obtained p = 0.000 <0.05, so it can be concluded that there is an effect of cigarette smoke exposure on the expression of Smad 3 and GDF9 in oocyte cells of Balb/c mice.

The lowest mean of apoptotic index expression in granulosa cells was found in the control group, while the highest mean was found in the treatment group. In the data normality test using *Shapiro Wilk*, it was found in the treatment group that data was normally distributed (p = 0.783). The results of statistical tests using *Mann Whitney* obtained p = 0.000 <0.05, so it can be concluded that there is an effect of cigarette smoke exposure on the apoptosis index in granulosa cells of Balb/c mice.

Examination of oocyte apoptosis in the control group and the treatment group, there was no apoptosis in the oocyte. The results of statistical tests using the *Mann Whitney* test obtained p = 1> 0.05, so it can be concluded that there is no effect of cigarette smoke exposure on the apoptosis index in oocytes of Balb/c mice.

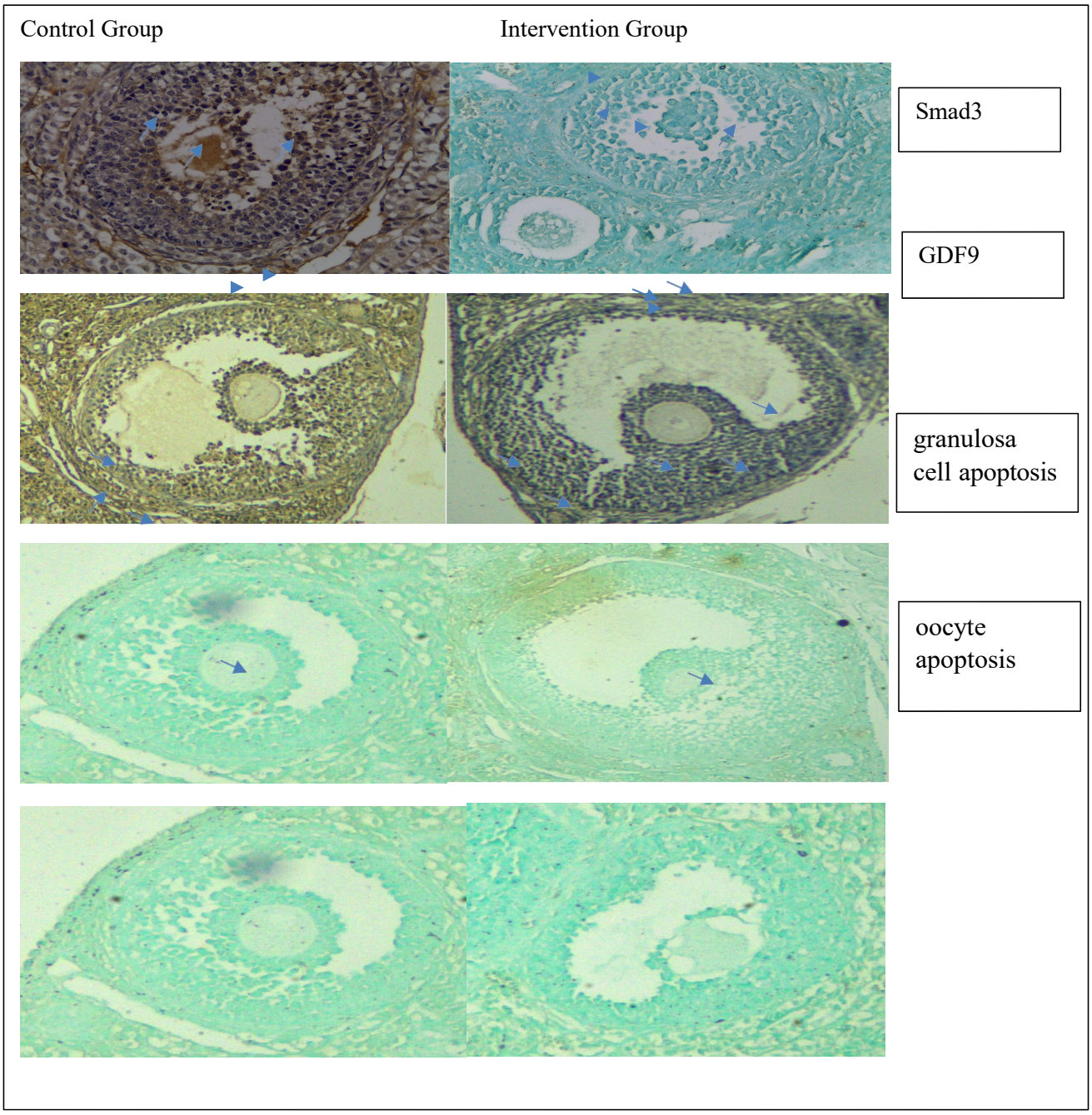

Figure 1. Smad3 expression, GDF9 expression, granulosa cell apoptosis, and oocyte apoptosis

Figure 1 showed that in control group Smad 3 expression in granulosa cells was positive, it was not seen in the cigarette smoke exposure treatment group that Smad 3 expression in granulosa cells is negative. Deficiency of Smad3 in mice will decrease fertilization because Smad3 plays role in follicle growth, aggression, and differentiation [11,12,18]. Administration of FSH to mice will express normal FSHR. This indicates that the interaction between Smad3 and FSH signals is involved in *downstream* FSHR in mouse ovaries[18].

The Smad signal pathway is critical for the transmission of the TGF-β signal family from cell surface to the nucleus. In the nucleus, Smad functions as a co-modulator of the transcription process for the regulation of TGF-β-dependent gene expression. Activin and inhibin are members of the TGF-β family. The difference between SMAD2 and SMAD3 proteins lies in the N-terminal region of MAD homology 1 (MH1), while SMAD2 contains 30 amino acids that are not in Smad3. The Smad2δexon3 protein occurs naturally due to the loss of this amino acid. Excessive expression of Smad2δexon3 will stimulate FSHβ mRNA in the same amount as stimulated by Smad3. Taken together, transcription activation of FSHβ will be stimulated by Smad3 and Smad2 in mouse gonadotroph cells[18].

Other evidence of Smad's involvement in FSH regulation can be seen from its role in the folliculogenesis process. The process of folliculogenesis itself is primarily responsible of FSH hormone so that the involvement of Smad in FSH regulation will indirectly affect the folliculogenesis

process[18,19]. Expression of Smad2 and Smad3 is involved during folliculogenesis. The Smad2 and Smad3 proteins are mediators for activin and TGF-β which are expressed in granulosa cells but not in large follicles. The expression of Smad2 is also in luteal cells. Smad2 protein is more responsive to activin stimulation while Smad3 is more responsive to TGF-β stimulation (Xu, 2002). Without Smad protein in large follicles, there is production of inhibin which acts as a paracrine factor that inhibits the growth of other follicles[11,19]. Giving inhibin to mouse ovaries in vitro will inhibit follicle growth and increase the percentage of granulosa cells that undergo apoptosis followed by an increase in Bax but not in Bcl-2[20]. The control mechanism of FSH secretion by inhibin is thought to be related to the blockade of activin's ability to produce FSHb through the Smad2 and Smad3 signal pathways[11,18,19,20]

In Figure 1 it can also be seen that in the control group the granulosa cell apoptosis index was negative, whereas in the smoke treatment group is positive. In experimental mice, it has been proven that the initial growth of follicles is triggered by gonadotrophin. The role of gonadotrophin in initiating resting follicles into the growth phase is considered to be most importance. The growth of this follicle is preceded by a change in the shape of granulosa cells from flat to cuboid. The change in these granulosa cells is influenced by what GnRH produces. The occurrence of apoptosis in follicles can be prevented by administering FSH and LH, but due to exposure to cigarette smoke, GnRH production decreases so that it will affects FSH and LH that made apoptosis in granulosa cells cannot be prevented (McGee & Hsueh, 2000).

Cigarette smoke exposure was shown that it can reduce the mean of GDF expression in 9 mice. The exposed group was lower than the control group. *Growth Differentiation Factor* (GDF-9) is only found in the ovaries and plays a role in maturation (Juengel, 2004). *Growth Differentiation Factor* (GDF-9) can stimulate granulosa cell proliferation. A deficiency of GDF-9 will cause the development of follicles stopped, without theca cell layer around the follicle, and ability of meiotic division in oocyte will be reduced [21] *Growth Differentiation Factor* (GDF-9) is affecting various functions of ovary cells including a decrease in cAMP so that the meiosis process can take place[21]. *Growth Differentiation Factor* (GDF-9) is synthesized by somatic ovum cells which directly affects the growth and function of oocytes. The presence and role of GDF-9 in oocytes are needed in the process of ovarian maturation and folliculogenesis. *Growth Differentiation Factor* (GDF-9) allowed cumulus expansion and an increase in LH receptors in granulosa cells. The mechanism of GDF-9 is to reduce cAMP so that it stimulates the expansion of the cumulus complex and produces hyaluronic acid, which indicates the oocyte has matured[21,22]. As a result of exposure to cigarette smoke, the expression of GDF9 was low, thereby affecting follicle maturation[22,23].

Based on the descriptive and analytical analysis carried out on the parameter of the number of oocytes *apoptotic* in the ovaries of mice exposed to cigarette smoke[24,25,26], it was found that there was no difference between the test groups. Based on the facts obtained in this study, exposure to cigarette smoke in female mice, both the control group and the cigarette smoke exposure group, had no apoptosis in oocyte cells[27,28,29]. However, in the treatment group, there was a cytoplasmic vacuole in the cigarette smoke exposure group, indicating necrosis. This indicated that exposure to cigarette smoke did not cause apoptosis in oocyte cells, but causes oocyte cells to experience necrosis, namely the apoptotic pathway through the necrosis pathway[30,31].

CONCLUSION

It can be concluded that cigarette smoke also decreased Smad3 expression in granulosa cells, SMAD 3 is a protein that mediates the transcription activity of estradiol in granulosa cells which influences oocyte development after estrogen receptor activation. Besides, exposure to cigarette smoke also increased apoptosis in granulosa cells. The presence of apoptosis in granulosa cells can cause folliculogenesis disorders in the ovaries. Cigarette smoke also decreased the expression of GDF 9 in oocytes, but there was no difference in the apoptosis in oocytes. It was possible that through necrosis, vacuolar was seen in oocytes.

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CONFLICT OF INTEREST

The authors state that there are no conflicts of interest regarding the publication of this article.

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ETHICAL CLEARANCE

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